Microbacterium koreense sp. nov., from sea water in the South Sea of Korea

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Microbacterium strains JS53-2^T and JS53-5 were isolated from sea water in the South Sea of Korea and subjected to phenotypic, chemotaxonomic and genetic characterization. The cells were found to be Gram-positive. These strains contained MK-11 and MK-12 as the main respiratory quinones and anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0} as the major cellular fatty acids. The DNA G + C content was 68 mol%. The cell-wall sugars of the isolates were galactose and xylose, and the diamino acid in the cell-wall hydrolysates was lysine. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the isolates represented an evolutionary lineage distinct from those of other *Microbacterium* species. DNA–DNA reassociation values between the isolates and reference strains *Microbacterium terregens* KCTC 19034^T, *Microbacterium lacticum* KCTC 9230^T, *Microbacterium aurum* KCTC 19091^T and *Microbacterium schleiferi* KCTC 19095^T were below 23%. On the basis of evaluation of the morphological, physiological and chemotaxonomic characteristics, 16S rRNA gene sequence comparisons and DNA–DNA hybridizations, a novel species, *Microbacterium koreense* sp. nov., is proposed for these isolates. The type strain is JS53-2^T (= KCTC 19074^T = CIP 108696^T = CCUG 50754^T).

The genus *Microbacterium* was first proposed as comprising a diverse collection of Gram-positive, non-spore-forming rods isolated during studies on lactic acid-producing bacteria by Orla-Jensen (1919). It was recognized as a very heterogeneous group. However, extensive systematic studies have resolved these relationships and emended the description of the genus *Microbacterium*. Its description was first emended in 1983 (Collins *et al.*, 1983) and then emended again in 1998 (Takeuchi & Hatano, 1998a). The genera *Microbacterium* and *Aureobacterium* are phylogenetically intermixed, although they are separate taxa on the basis of (primarily) cell-wall differences. The presence of the L-diamino acid L-lysine in the cell wall is characteristic of *Microbacterium* and the presence of the D-diamino acid D-ornithine in the cell wall is characteristic of *Aureobacterium*. The other chemotaxonomic and phenotypic characteristics of the members of the two genera have shown very similar profiles. On the basis of these data, Takeuchi & Hatano (1998a) emended the descriptions of the genera *Microbacterium* and *Aureobacterium*, uniting them in a redefined genus *Microbacterium*.

Members of the genus *Microbacterium* are found in various environments, including soil, water, plants, milk products and human clinical samples. During the course of our studies on microbial diversity in the sea water of Korea, we found yellow-pigmented bacteria in sea water from the South Sea of Korea (the Korea Strait); experiments were performed to determine the morphological, biochemical and phylogenetic characteristics of those isolates. On the basis of the results, we propose that these isolates be assigned to a novel species.

Strains JS53-2^T and JS53-5 were isolated (on tryptic soy agar) from sea water from the South Sea of Korea. The isolates and reference strains *Microbacterium terregens* KCTC 19034^T (= IFO 12961^T), *Microbacterium lacticum* KCTC 9230^T (= DSM 20427^T), *Microbacterium aurum* KCTC 19091^T (= IFO 15204^T) and *Microbacterium schleiferi* KCTC 19095^T (= IFO 15075^T) were cultured on tryptic soy agar for 48 h at 30 °C.

The cell morphology was examined by using light microscopy. Motility was determined with an optical microscope, using the hanging drop technique (Skerman, 1967). Anaerobic growth was recorded in an anaerobic chamber (CO₂/H₂/N₂, 7:7:86; Forma Scientific) on tryptic soy agar for up to 1 week. Growth in the presence of various concentrations of added NaCl (0.5, 1, 2, 5, 10, 15 and 20%, w/v) was tested with nutrient broth (Difco) as the basal medium. Growth at different temperatures was observed in trypticase soy broth at 10, 15, 20, 25, 30, 37, 42 and 50 °C: 10 ml trypticase soy

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Photomicrographs of strain JS53-2^T, an extended phylogenetic tree and a table of whole-cell fatty acid profiles are available as supplementary material in IJSEM Online.

broth was adjusted to pH values ranging from 4.0 to 11.0 (100 mM citric acid/200 mM Na₂HPO₄ at pH 4.0–5.0, 100 mM Na₂HPO₄/NaH₂PO₄ buffer at pH 6.0–8.0, 100 mM NaHCO₃/Na₂CO₃ buffer at pH 9.0–10.0 and 50 mM Na₂HPO₄/100 mM NaOH buffer at pH 11.0), using a method based on that of Yumoto *et al.* (1998). Growth was estimated by monitoring the OD₆₀₀. Physiological and

biochemical characteristics were determined using API 20E, API 20NE and API 50 CHB kits (bioMérieux). All API tests were performed in accordance with the manufacturer's instructions. Catalase activity was determined by means of bubble production from 3 % (v/v) H₂O₂, while oxidase activity was determined using 1 % (w/v) tetramethyl-*p*-phenylenediamine.

Table 1. Distinctive characteristics of the isolates and related *Microbacterium* species

Strains: 1, *M. lacticum* KCTC 9230^T; 2, *M. terregens* KCTC 19034^T; 3, *M. aurum* KCTC 19091^T; 4, *M. schleiferi* KCTC 19095^T; 5, *M. koreense* JS53-2^T and JS53-5. Data are from this study, from Takeuchi & Hatano (1998a, b) and from Behrendt *et al.* (2001). Abbreviations: +, positive; w, weakly positive; –, negative. For all strains, the results are positive for growth in 0.5 % NaCl, aesculin hydrolysis and acid production from glucose, fructose, mannose, mannitol, cellobiose and maltose; all strains are negative for motility, gelatin hydrolysis and oxidase.

Characteristic	1	2	3	4	5
Colony colour	Yellow	Yellow	Yellow	Yellow	Light yellow
Anaerobic growth	+	–	+	–	–
Catalase	+	–	+	+	+
Temperature range (°C)	15–30	20–30	10–42	10–42	20–37
Growth in NaCl at 5 %	–	–	+	–	+
H ₂ S production	+	–	–	–	–
Acid production from:					
Glycerol	+	–	–	w	–
D-Xylose	–	+	–	+	w
Methyl β-D-xyloside	–	+	–	–	–
Galactoside	+	–	+	+	w
Rhamnose	–	–	+	–	+
N-Acetylglucosamine	+	–	+	–	+
Arbutin	+	–	–	–	–
Salicin	+	–	–	–	–
Lactose	+	–	–	w	–
Melibiose	–	+	–	–	–
Sucrose	–	+	+	+	+
Trehalose	–	+	+	+	w
Starch	+	–	+	–	+
Raffinose	–	+	+	–	–
Glycogen	+	–	–	w	w
Assimilation of:					
Glucose	+	–	+	–	+
Mannose	+	–	+	–	+
Mannitol	+	–	+	–	+
N-Acetylglucosamine	+	–	w	–	+
Maltose	–	–	+	–	+
DNA G + C content (mol%)	71	68	69.2	68	68
Menaquinones	MK-11, MK-12	MK-12, MK-13	MK-11, MK-12	MK-10, MK-11, MK-12	MK-11, MK-12
Cell-wall diamino acid*	Lys	Orn	Lys	Orn	Lys
Cell-wall sugars†	Rha, Man, Gal	Rha, Gal, 6dTal	Fuc, Gal, Glc	6dTal	Gal, Xyl

*Lys, Lysine; Orn, ornithine.

†6dTal, 6-Deoxytalose; Fuc, fucose; Gal, galactose; Man, mannose; Rha, rhamnose; Xyl, xylose.

The isolates were aerobic, non-motile, rod-shaped and Gram-positive. In old cultures, the rods became shorter or spherical (see Supplementary Fig. S1 available in IJSEM Online). Colonies were circular, convex with entire margins, moist, shiny and light yellow in colour. The isolates grew neither in an anaerobic chamber at 37 °C nor with NaCl at concentrations above 10 %. Optimum growth was observed without added NaCl. The isolates grew at 20–37 °C and at pH 6.0–8.0. The optimum growth temperature and pH were 30 °C and pH 7. The morphological, physiological and chemotaxonomic characteristics of the isolates are different from those of phylogenetically closely related *Microbacterium* species (Table 1).

For total analysis of the cellular fatty acids, the cells were cultured on tryptic soy agar for 48 h at 30 °C and then the fatty acids were extracted according to the Microbial Identification System instructions, as described previously (Lee *et al.*, 1996; Yang *et al.*, 1993). The isoprenoid quinones were extracted with chloroform/methanol (2 : 1, v/v) and purified by using TLC on Merck Kieselgel 60 F₂₅₄ plates (20 × 20 cm, 0.5 mm thickness) using petroleum ether/diethyl ether (9 : 1, v/v) as the solvent. The identities of the quinones were determined by using reversed-phase HPLC analysis, as described by Shin *et al.* (1996). The whole-cell sugars were determined by using the method of Komagata & Suzuki (1987). Analysis of the diamino acid in peptidoglycan hydrolysates was carried out as described by Bousfield *et al.* (1985) on a cellulose TLC plate.

The whole-cell fatty acid profiles for the strains are shown in Supplementary Table S1 (available in IJSEM Online). The major fatty acids in the isolates and in related *Microbacterium* species were anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. The cell-wall sugars and the major menaquinones of the isolates were found to be galactose and xylose and MK-11 and MK-12, respectively. Differential characteristics of the isolates and reference strains are shown in Table 1. The diamino acid in cell-wall hydrolysates of the isolates and reference strains *M. lacticum* KCTC 9230^T and *M. aurum* KCTC 19091^T was lysine, while that of reference strains *M. terregens* KCTC 19034^T and *M. schleiferi* KCTC 19095^T was ornithine.

DNA was extracted and purified by using a modified version of the method described by Marmur (1961). The G+C content of the DNA was determined using the HPLC method described by Tamaoka & Komagata (1984). The DNA G+C contents of the isolates and reference strains *M. lacticum* KCTC 9230^T, *M. terregens* KCTC 19034^T, *M. aurum* KCTC 19091^T and *M. schleiferi* KCTC 19095^T were 68, 71, 68, 69 and 68 mol%, respectively.

Two universal primers (9F and 1492R) described by Stackebrandt & Liesack (1993) were used for PCR amplification of the 16S rRNA gene, and the amplified PCR product was purified using a QIAquick PCR purification kit (Qiagen). The purified 16S rRNA gene was then sequenced using an ABI Prism BigDye Terminator cycle sequencing

ready reaction kit (Applied Biosystems), with an automatic DNA sequencer (model 377; Applied Biosystems). Nearly complete 16S rRNA gene sequences (1435 bp) were determined for isolates JS53-2^T and JS53-5, and aligned with the 16S rRNA gene sequences of representatives of the genus *Microbacterium* and related taxa, using CLUSTAL W software (Thompson *et al.*, 1994). A phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) based on distance-matrix data. Evolutionary distances were calculated using the model of Jukes & Cantor (1969). The PHYLIP software package (Felsenstein, 1993) was used for all analyses. The topology of the phylogenetic tree was evaluated using a bootstrap analysis (Felsenstein, 1985) of the neighbour-joining method, based on 1000 replications. The 16S rRNA gene sequences of the strains were compared with those of closely related reference strains. A phylogenetic tree indicated that isolates JS53-2^T and JS53-5 belong to the genus *Microbacterium* (Fig. 1; an extended version of this tree is available as Supplementary Fig. S2 in IJSEM Online).

Isolates JS53-2^T and JS53-5 had identical 16S rRNA gene sequences and morphological, physiological and chemotaxonomic characteristics, implying that they might be members of the same species. They were phylogenetically closely related to *M. lacticum* DSM 20427^T (98.1 % sequence similarity), *M. schleiferi* IFO 15075^T (98.1 % sequence similarity), *M. terregens* IFO 12961^T (97.9 % sequence similarity) and *M. aurum* IFO 15204^T (97.4 % sequence similarity).

Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequences, showing the position of isolates JS53-2^T and JS53-5 with respect to other species from genus *Microbacterium* and related taxa. *Arthrobacter globiformis* served as the external reference. Numbers indicate bootstrap values. Bar, 0.01 accumulated changes per nucleotide. An extended version of this tree is available as Supplementary Fig. S2 in IJSEM Online.

Table 2. DNA–DNA reassociation values for the isolates and related *Microbacterium* species

Strain	Reassociation (%) with labelled DNA from:					
	1	2	3	4	5	6
1. JS53-2 ^T	100	102	18	21	15	20
2. JS53-5	93	100	22	14	15	17
3. <i>M. lacticum</i> KCTC 9230 ^T	20	16	100	15	20	25
4. <i>M. terregens</i> KCTC 19034 ^T	19	23	27	100	21	13
5. <i>M. aurum</i> KCTC 19091 ^T	19	13	18	14	100	22
6. <i>M. schleiferi</i> KCTC 19095 ^T	12	16	19	25	29	100

The levels of sequence similarity between these strains and other *Microbacterium* species ranged from 93.2 to 98.1 %.

DNA–DNA hybridization was carried out by means of fluorometric hybridization in microdilution wells, using biotinylated DNA (Ezaki *et al.*, 1989). As shown in Table 2, the DNA–DNA reassociation values between isolates JS53-2^T and JS53-5 and reference strains *M. lacticum* KCTC 9230^T, *M. terregens* KCTC 19034^T, *M. aurum* KCTC 19091^T and *M. schleiferi* KCTC 19095^T were less than 23 %. The phylogenetic definition of a species generally involves ‘strains with approximately 70 % or greater DNA–DNA relatedness’ (Wayne *et al.*, 1987). According to currently available data, organisms with a sequence similarity of less than 97.0 % will not reassociate to more than 60 %, no matter which hybridization method is applied (Stackebrandt & Goebel, 1994; Rossello-Mora & Amann, 2001; Stackebrandt *et al.*, 2002). Therefore, the phylogenetic and DNA–DNA hybridization results from this work demonstrate that the isolates are phylogenetically closer to members of the genus *Microbacterium* and are not related to any previously described *Microbacterium* species at the species level.

On the basis of the above results, a novel species, *Microbacterium koreense* sp. nov., is proposed.

Description of *Microbacterium koreense* sp. nov.

Microbacterium koreense (ko.re.en'se. N.L. neut. adj. *koreense* pertaining to Korea, where the type strain was isolated).

Aerobic, non-motile, Gram-positive organism. In young cultures, cells are rods about 0.7 µm in width by 3.0–4.0 µm in length. In old cultures, rods become shorter or spherical. Colonies are circular, convex with entire margins, moist, shiny and light yellow in colour. Does not grow in an anaerobic chamber at 37 °C. The temperature range for growth is 20–37 °C, with optimal growth at 30 °C. The pH range for growth is 6.0–8.0, with optimal growth at pH 7.0. No growth occurs at an NaCl concentration of more than 10 %; optimal growth occurs without added NaCl. Strains give positive results for aesculin hydrolysis, for catalase and for acid production from glucose, fructose, mannose, rhamnose, mannitol, *N*-acetylglucosamine, cellobiose, maltose, sucrose and starch. Weakly positive for acid

production from D-xylose, galactoside, trehalose, glycogen and D-turanose. Negative for oxidase, nitrate reduction, indole production, glucose acidification, arginine hydrolysis, urease, gelatin hydrolysis, β-galactosidase and acid production from glycerol, erythritol, D-arabinose, L-arabinose, ribose, L-xylose, adonitol, methyl β-D-xyloside, sorbose, dulcitol, inositol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, amygdalin, arbutin, salicin, lactose, melibiose, inulin, melezitose, raffinose, xylitol, gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate. The G + C content of the DNA is 68 mol%, the major isoprenoid quinones are MK-11 and MK-12, and the major cellular fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. The cell-wall sugars are galactose and xylose. The diamino acid in the cell-wall hydrolysates is lysine.

The type strain is JS53-2^T (= KCTC 19074^T = CIP 108696^T = CCUG 50754^T), isolated from sea water in the South Sea of Korea (the Korea Strait).

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References

- Behrendt, U., Ulrich, A. & Schumann, P. (2001). Description of *Microbacterium foliorum* sp. nov. and *Microbacterium phyllosphaerae* sp. nov., isolated from the phyllosphere of grasses and the surface litter after mulching the sward, and reclassification of *Aureobacterium resistens* (Funke *et al.* 1998) as *Microbacterium resistens* comb. nov. *Int J Syst Evol Microbiol* 51, 1267–1276.
- Bousfield, I. J., Keddle, R. M., Dando, T. R. & Shaw, S. (1985). Simple rapid methods of cell wall analysis as an aid in the identification of aerobic coryneform bacteria. In *Chemical Methods in Bacterial Systematics*, vol. 20, pp. 221–236. Edited by M. Goodfellow & D. E. Minnikin. London: Academic Press.
- Collins, M. D., Jones, D., Keddle, R. M., Kroppenstedt, R. M. & Schleifer, K. H. (1983). Classification of some coryneform bacteria in a new genus *Aureobacterium*. *Syst Appl Microbiol* 4, 236–252.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid–deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in

which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Felsenstein, J. (1993). PHYLIP (phylogeny inference package), version 3.5c. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

Komagata, K. & Suzuki, K. (1987). Lipid and cell-wall analysis in bacterial systematics. *Methods Microbiol* **19**, 201–203.

Lee, J.-S., Jung, M.-C., Kim, W.-S. & 10 other authors (1996). Identification of lactic acid bacteria from kimchi by cellular FAMES analysis. *Korean J Appl Microbiol Biotechnol* **24**, 234–241.

Marmur, J. (1961). A procedure for the isolation of DNA from microorganisms. *J Mol Biol* **3**, 208–218.

Orla-Jensen, S. (1919). *The Lactic Acid Bacteria*. Copenhagen: Host & Sons.

Rossello-Mora, R. & Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiol Rev* **25**, 39–67.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Shin, Y. K., Lee, J.-S., Chun, C. O., Kim, H.-J. & Park, Y.-H. (1996). Isoprenoid quinone profiles of the *Leclercia adecarboxylata* KCTC 1036^T. *J Microbiol Biotechnol* **6**, 68–69.

Skerman, V. B. D. (1967). *A Guide to the Identification of the Genera of Bacteria*, 2nd edn. Baltimore: Williams & Wilkins.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Stackebrandt, E. & Liesack, W. (1993). Nucleic acids and classification. In *Handbook of New Bacterial Systematics*, pp. 152–189. Edited by M. Goodfellow & A. G. O'Donnell. London: Academic Press.

Stackebrandt, E., Frederiksen, W., Garrity, G. M. & 10 other authors (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**, 1043–1047.

Takeuchi, M. & Hatano, K. (1998a). Union of the genera *Microbacterium* Orla-Jensen and *Aureobacterium* Collins *et al.* in a redefined genus *Microbacterium*. *Int J Syst Bacteriol* **48**, 739–747.

Takeuchi, M. & Hatano, K. (1998b). Proposal of six new species in the genus *Microbacterium* and transfer of *Flavobacterium marinotypicum* ZoBell and Upham to the genus *Microbacterium* as *Microbacterium maritypicum* comb. nov. *Int J Syst Bacteriol* **48**, 973–982.

Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed phase high-performance liquid chromatography. *FEMS Microbiol* **25**, 125–128.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches of bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Yang, P., Vauterin, L., Vancaneyt, M., Swings, J. & Kersters, K. (1993). Application of fatty acid methyl esters for the taxonomic analysis of the genus *Xanthomonas*. *Syst Appl Microbiol* **16**, 47–71.

Yumoto, I., Yamazaki, K., Sawabe, T., Nakano, K., Kawasaki, K., Ezura, Y. & Shinano, H. (1998). *Bacillus horti* sp. nov., a new Gram-negative alkaliphilic bacillus. *Int J Syst Bacteriol* **48**, 565–571.